"Spectroscopic study of the interaction of actinomycin D with oligonucleotides carrying the central base sequences XGCY- and XGGCCY- using multivariate methods." Vives, M., Tauler, R., Eritja, R., Gargallo, R. Anal. Bioanal. Chem., 387(1), 311-320 (2007).

PMID: 17123067, doi: 10.1007/s00216-006-0946-8

# Spectroscopic study of the interaction of actinomycin D with oligonucleotides carrying the central base sequences –XGCY- and –XGGCCY- using multivariate methods

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## 1. Abstract

The interaction of actinomycin D (ACTD) with the oligonucleotides 5'-CAAAGCTTTG-3', 5'-CATGGCCATG-3' and 5'-TATGGCCATA-3' were studied by means of acid-base titrations, and mole-ratio and melting experiments monitored by molecular absorption and circular dichroism (CD) spectroscopies. For each experiment, CD and molecular absorption spectra were recorded at each point of the experiment, and later analyzed by means of appropriate multivariate data analysis methods. The study of the interaction of these oligonucleotides with ACTD at 25 oC showed the formation of an interaction complex with a stoichiometry 1:1 (ACTD:duplex) and log of formation constants 5.1 ± 0.3, 6.4 ± 0.2, and 5.6 ± 0.2, respectively. An additional interaction complex at higher temperatures has been detected in the case of AGCT and GGCC_G sequences, which has been related with the formation of a hairpin structure stabilized by the terminal G-3' base.



*Keywords*: actinomycin D, DNA, multivariate analysis, MCR-ALS

## 2. Introduction

Actinomycin D (ACTD) (Scheme 1) is one of the most studied DNA-binding ligands, because of its function as a model sequence-specific antitumor antibiotic. It consists of a planar 2-aminophenoxazin-3-one chromophore ring with two cyclic pentapeptide lactones. This drug exerts its biological function via inhibition of transcription. It binds to duplex DNA via intercalation with high affinity and it dissociates slowly from DNA [1]. The binding of ACTD to DNA is sequence-specific and it has been shown to prefer the duplex GpC site. This base-sequence specificity derives mainly from the formation of strong hydrogen bonds in the minor groove, which suggest that the binding characteristic of ACTD to a GpC site may likely be affected by the adjacent flanking base pairs. A large number of studies have been done dealing with the interaction of ACTD with a large variety of DNA sequences [2-5]. These works have revealed the mechanism of the interaction at low temperatures, which is mainly related to the central sequence –XGCY-. Some authors have also proposed the existence of an additional interaction of ACTD with single stranded DNA at high temperatures [1]. This mode of interaction could be related to a hairpin conformation of DNA carrying the central base sequence –XGGCCY-. Previous studies have suggested the formation of a complex between ACTD and a hairpin conformation of oligonucleotides carrying the sequence CXYGGCCY'X'G [1]. This complex would be formed at temperatures higher than those at which would the complex duplex:ACTD is formed, because it would be necessary the breaking of most of the Watson-Crick base pairing to yield the hairpin. According to this hypothesis, only oligonucleotides carrying the G-3' base pair would form such structure.

Traditionally, ACTD interaction with DNA has been studied by means of mole-ratio and melting experiments monitored at that wavelength where the most dramatic spectral changes could be observed [6, 7]. This procedure, which is known as an univariate approach in Statistics and Chemometrics, has some advantages, like the simplicity of data analysis. However, when analyzing data recorded in the study of complex systems like those showing a spectral overlap, the reliability of the results obtained by this approach it is difficult to assess. In these cases, recording the spectral signal in a whole wavelength range and later application of appropriate data analysis methods could

be a valid alternative. This procedure, which is known as an multivariate approach, has also some advantages and drawbacks. Thus, a successful resolution of data recorded when monitoring the chemical behavior of complex systems can be achieved. On the other hand, the mathematical tools needed to carry out multivariate analysis are usually more complex than those applied in univariate analysis. For a review about the applications of multivariate data analysis methods to biochemistry and biophysics studies, see [8].

In this work, we show the results obtained in the study the interaction of ACTD with some oligonucleotides using a multivariate approach. The objectives are the characterization of the acid-base and temperature-induced transitions of these oligonucleotides and of their interaction complexes with ACTD, in addition to the calculation of the formation constants at 25 $^{o}$C and pH 7. The possible formation of interaction complexes between ACTD and the single-stranded forms of these sequences will be also investigated. Three DNA sequences have been chosen for this study: 5'-CAAAGCTTTG-3' (AGCT), 5'-CATGGCCATG-3' (GGCC_G) and 5'-TATGGCCATA-3' (GGCC_A), including the central base pairs sequences: -AGCT-, -GGCC-XYG, and -GGCCXYA-. To achieve the described objectives, acid-base titrations and melting and mole-ratio experiments have been carried out by measuring CD and molecular absorption spectra. The data sets have been analyzed by means of appropriate multivariate data analysis methods.

## 3. Results

### 3.1. Preliminary study of the oligonucleotides

Before the study of the interaction with ACTD, the acid-base properties and the melting process of isolated oligonucleotides were studied. First, several acid-base titrations of solutions containing each one of the oligonucleotides were spectroscopically monitored. Figure 1a shows the experimental CD and absorption data recorded along the acid-base titration of AGCT. These spectra were collected from pH 11.86 to pH 1.80, and arranged in an augmented data matrix [$\mathbf{D_{CD}}$,$\mathbf{D_{abs}}$], with 29 rows (spectra) and 562 columns (i.e., 281 wavelengths measured in each spectroscopy). Application of EQUISPEC allowed the calculation of the number $N$ of acid-base species, and of

their concentration **C** and spectral $\mathbf{S}^T$ profiles. For this system, $N = 3$, i.e., only two pKa values were determined from the data set, corresponding to 4.1 ± 0.6 and 10.4 ± 0.6 (Figure 1b). The first $pK_a$ value corresponds to the deprotonation of cytosine and adenine bases, while the second $pK_a$ value corresponds to the deprotonation of thymine and guanine bases. The resolved concentration profiles **C** show clearly that near pH 7 only one acid-base species must be considered. From the resolved $\mathbf{S}^T$ spectra (Figure 1c and 1d), it is clear that species 2, that corresponding to the neutral oligonucleotide, shows the higher CD signal, which is related to the higher degree of order of this species. The resolved molecular absorption spectra show, as expected, the molar absorptivity of the neutral species being lower than those for the other two species do. The acidic species shows a shift to 280 nm, which is characteristic of protonation of cytosines. A similar study was carried out for GGCC_G and GGCC_A, giving similar $pK_a$ values to those determined for AGCT. Similar trends to those described for the resolved spectra of AGCT were also observed for these two oligonucleotides.

Melting experiments for each oligonucleotide were also carried out and, as in the acid-base study, the whole CD and molecular absorption spectra were analyzed. However, in this case, spectroscopic data were analyzed by means of MCR-ALS, since hard-modeling methods are still not available for the analysis of multivariate melting data. In all cases, results showed that only two conformations were present in the temperature range studied, which were related to the initial ordered conformation and to the final random coil conformation. Analysis of residuals (matrix **E** in Equation 1) did not show the presence of any additional conformation. Table 1 summarizes the $T_m$ values determined from the resolved concentration profiles for each oligonucleotide. In addition, Figure 2 shows the results obtained for the melting of GGCC_G. From the resolved concentration profiles, it is clear that at 25 $^oC$ duplex is the predominant conformation. The resolved CD and molecular absorption spectra show the expected trends for the melting of a decamer. In the case of GGCC_G, CD bands shifts from 244 and 268 nm to 241 and 277 nm, respectively, upon heating, while signal decreases. The position and intensity of the bands for the ordered conformation of GGCC_G were not exactly those expected for a standard B-DNA (approximately equal positive,

275 nm, and negative, 245 nm, components centered around 260 nm) [9]. This observation agrees with previous works, where the conformation of the –GGCC- sequence in GGCC_G was suggested to exist predominantly in a non-standard B-conformation [1].

### 3.2. Preliminary study of ACTD

The acid-base study of ACTD was carried out in a similar way to that above described. In this case, however, no spectral changes were observed neither in CD nor in molecular absorption data due to pH changes (from 4 to 9). This observation agrees with an early work of Cavalieri and Neunchin [10], where no spectral changes were observed between pH 5 and 10. Studies of electrophoretic mobility of ACTD carried out by these authors did not show electrophoretic mobility between pH 2.7 and 10. Therefore, these authors concluded that there was no structural changes were present on the ACTD molecule in the studied pH range. Bibliographic search revealed that few works have been published in relation to the acid-base properties of ACTD since that early work. Recently, Szczepanik *et al.* have reported a $pK_a$ value for the amino group in ACTD (8.06) determined from potentiometric studies [5]. As described above, no spectral changes were observed upon pH changes in this work and, therefore, it has been assumed that only one acid-base spectroscopically active species of ACTD was present in the pH range studied.

In addition to acid-base study of ACTD, the spectral behavior upon heating was investigated. While absorption spectra did not show clear changes, CD spectra change slightly (Figure 3). As in the case of oligonucleotides, the whole set of spectroscopic data was analyzed by MCR-ALS. Two conformations were present along the melting, which could be related to a different degree of order in the ACTD molecule. The concentration profiles showed a non-sharp profile, characteristic of transitions lacking of cooperative effects. The resolved CD spectra showed a shift of the negative band at 265 to 250 nm, of the positive band at 297 to 295 nm and, finally, the disappearance of the negative band at 378 upon heating. For a complex molecule like ACTD, it is very difficult to make unequivocal assignments of each optically active band. Probably, the structural changes observed could be related to a partial loss of structure in the peptide ring. In this sense, Ziffer et al. [11]

proposed the influence of lactone rings into the optically active band at 380 nm. Changes in this region upon heating could be related to changes in the lactone ring structure. Another possible explanation for the changes reflected by CD spectroscopy could be the presence of a dimer – monomer equilibrium. The theoretical percentages of monomer and dimer species were calculated from the value of $K_{dimer} = 1 \cdot 10^3\ M^{-1}$ calculated by Crothers [12]. For a 23 μM ACTD solution, 96 % should be present as monomer and 4 % as dimer. A similar value for $K_{dimer}$ is $1.4 \cdot 10^3\ M^{-1}$ (at 18 $^oC$) was calculated by Angerman et al. from NMR measurements [13].

### 3.3. Study of the interaction of ACTD with the oligonucleotides

After the preliminary study of the acid-base properties and melting process of each oligonucleotide and ACTD, their interactions were investigated. From the previous acid-base studies of oligonucleotides, it was clear that only the neutral species of each oligonucleotide was present in the pH range 6 - 8. A similar conclusion was drawn for ACTD. Thus, the interaction complex at pH 7 will be formed by the neutral form of each oligonucleotide and ACTD. Table 1 summarizes the values of the formation constants (log β) determined for each system from acid-base titrations and mole-ratio experiments. As example, Figure 4 shows the results obtained from the analysis by EQUISPEC of the spectroscopic data recorded along a mole-ratio experiment of the mixture GGCC_G:ACTD. In this case, the resolved CD spectrum (Figure 4c) for the interaction complex shows different characteristics to those of GGCC_G or ACTD. Thus, a small shift of the negative band centered at 375 (in the spectrum of ACTD) to 378 nm (in the spectrum of the complex) can be observed. In addition, a shift of the negative weak band centered at 450 (in the spectrum of ACTD) to 465 nm (in the spectrum of the complex). These facts can be related to interactions of the base pairs in the oligonucleotide with the benzenoide portion of ACTD (for the 380 nm band), and with the quinoid portion (for the 470 nm band) [14]. Moreover, positive (224 and 292 nm) and negative (246 nm) bands can be observed in the CD spectrum of the complex. On the other hand, the resolved pure molecular absorption spectrum of the interaction complex shows a batochromic shift from 442 (in ACTD) to 465 nm.

Table 1 summarizes the $T_m$ values determined from MCR-ALS analysis of spectroscopic data recorded along melting of mixtures DNA:ACTD. All three sequences show three transitions. As example, MCR analysis of the data recorded along the melting experiment of a mixture GGCC_G:ACTD ($C_{actino2}$ = 12 μM, $C_{ACTD}$ = 9 μM, pH 7) needed four components to be described properly, yielding $T_m$ values of 41, 53 and 69 ± 1 $^oC$, respectively (Figure 5). When a lower number of components was considered, large residuals were obtained. The resolved concentration profile reveals a complex system where more than one interaction complex is formed. The spectrum of the first component shows the characteristics of the interaction complex previously resolved in mole-ratio studies, such as a large amplitude of the negative CD band at 250 nm or the batochromic shift of the visible absorption band. Therefore, this component has been explained as the mixture of the complex GGCC_G:ACTD, and free GGCC_G and ACTD. The positive CD band at 270 nm has been related to the duplex. The spectrum of the second component shows little changes in absorption bands but a remarkable decrease in the amplitude of the CD band at 250 nm and a concomitant increase in the amplitude of the band at 272 nm. On the other hand, the position and shape of the absorption and CD bands in the visible region still denote the existence of complexed ACTD. The spectrum of the third component shows again a CD band reflecting the formation of a complex. The loss of the band at 272 nm could reflect the disappearance of duplex. Finally, the last spectrum can be easily related to the presence of both free drug and GGCC_G, without any interaction, as observed in the position and shape of the visible bands in absorption and CD techniques. Taking into account the information provided by the resolved pure spectra, the concentration profile is now explained as follows. The first transition would correspond to the melting of GGCC_G not bonded to ACTD. The value of $T_m$ for this transition is lower than that previously determined (49 $^oC$) and this is explained because the small concentration of free GGCC_G. The second transition would correspond to the denaturation of the weak GGCC_G:ACTD complex. The last transition would correspond to the disruption of the stronger complexes formed once GGCC_G duplex has melted to single strands.

The study of the melting behavior of AGCT:ACTD and GGCC_A:ACTD mixtures yield similar characteristics to those above described for the GGCC_G:ACTD mixtures. In other words, the resolved concentration profiles and pure spectra seem to confirm the presence of an additional interaction complex at high temperatures. Contrary to the melting of GGCC_G and AGCT, the study of the thermal denaturation of GGCC_A revealed the formation of a weak duplex:ACTD complex, as reflected in the small shift of $T_m$ values for the duplex (43 $^oC$) and for the complex (46 $^oC$).

## 4. Discussion

The work presented here describes the application of a multivariate approach for the study of the interaction between ACTD and several oligonucleotides. First, a preliminary study of acid-base and melting properties of each DNA sequence and ACTD was done. From the results obtained in the acid-base study, it is worth to comment the absence of cooperative effects along the protonation / deprotonation process, as denoted by the successful application of a very simple acid-base model based on two acid-base constants. The absence of cooperative effects can be also observed in the smooth shape of the resolved concentration profiles. This fact is related to the short DNA sequences studied in this work. For longer oligonucleotides or natural DNAs, the application of such a simple model would not be possible, and more complex models or soft-modeling methods, like MCR-ALS, would be required. In those cases, sharp concentration profiles would have been finally obtained. Melting studies of oligonucleotides yield similar values to those previously described by other authors. Thus, the $T_m$ value determined for AGCT (5'-CAAAGCTTTG-3') is similar to that previously determined for the similar sequence 5'-CATAGCTATG-3' (35 $^oC$, [3]) at slightly different conditions (0.1 M NaCl, pH 8). The Tm value determined for GGCC_G is slightly higher than for AGCT due to the presence of additional G•C base pairs, and it is in good agreement with the value previously reported by Chen et al. (47 $^oC$, [1, 3]) at pH 8.0 and 0.1 M NaCl. Finally, the $T_m$ value determined for GGCC_A (5'-TATGGCCATA-3') agrees with that previously determined (41 $^oC$, [3]).

Acid-base titrations and mole-ratio experiments have been used to determine the existence of an interaction complex between each DNA sequence and ACTD at 25 $^{o}$C and pH 7.0. Table 1 summarizes the determined values for the formation constants. Overall, similar values were obtained by other authors, such as Chu *et al.* for the titration of ACTD with d(GAAGCTTC)$_2$ (log β= 6.1 $M^{-1}$, pH 8, 45 mM Tris-HCl, 1.25 mM $MgCl_2$, 10 $^{o}$C) [15], or Chen *et al.* for the d(ATCAGCAGAT)$_2$ (log β 6.0 $M^{-1}$, pH 8, 0.1 M NaCl, 1 mM $MgCl_2$, 20 $^{o}$C) [4]. From the results summarized in Table 1, it is clear that oligonucleotides carrying the central sequence GGCC form the most stable interaction complex with ACTD at 25 $^{o}$C, especially GGCC_G.

Comparison of the melting behavior for the DNA:ACTD mixtures reveals some interesting facts. The determined $T_m$ values from the resolved concentration profiles suggest the formation of two interaction complex between ACTD and the duplex and single-stranded forms of the DNA sequences studied, respectively. The existence of the ACTD:duplex complex and the corresponding $T_m$ values determined for the melting of these complexes agree with those previously determined for similar sequences by Chen *et al*. However, the formation of a complex between ACTD and the single stranded forms had only been described for the GGCC_G sequence. In all cases, the interaction complex between the drug and the single stranded form seems to be stronger than the duplex:ACTD complex. Previous studies had proposed the formation of a hairpin structure for GGCC_G where the terminal G-3' base folds back to base-pair with the C base of GGC [1].

Summarizing, the results presented here show the application of a multivariate approach for the study of the interaction of drugs with DNA. In addition to the duplex:ACTD complex at low temperatures, the results suggest the formation of an additional interaction complex at high temperature.

# 5. Experimental section

## 5.1. Reagents

Sequences 5'-CAAAGCTTTG-3' (AGCT), 5'-CATGGCCATG-3' (GGCC_G) and 5'-TATGGCCATA-3' (GGCC_A) were prepared on 1 μmol scale using standard 2-cyanoethyl phosphoramidites (Cruachem Ltd.). Syntheses were performed using an automatic DNA synthesizer (Applied Biosystems Mod.392). Sequences were deprotected using standard protocols (concentrated ammonia, 55 $^{o}$C, overnight). After deprotection, oligonucleotides were desalted using Sephadex G-25 columns. The oligonucleotide (as single strand) concentration was determined by UV absorbance measurements (260 nm) after melting, using the following extinction coefficients: 7600, 8700, 12160, 15340 $M^{-1}$ $cm^{-1}$ for C, T, G and A [17], respectively. Oligonucleotide samples were kept at 4 $^{o}$C until their use. ACTD was purchased from Sigma (St. Louis, USA). Stock solutions were prepared by dissolution of the solid reagent into a volume of water, followed by filtration through a 0.45 μm filter. Concentrations of the diluted ACTD solutions were determined by measuring absorbances at 440 nm using extinction coefficient of 24450 $M^{-1}$ at 25 $^{o}$C.

Diluted solutions for measurements were prepared in Ultrapure water (Millipore) with the appropriate amounts of NaCl (Merck, a.r.), $MgCl_2$ (Probus, a.r.), and the buffer compound $Na_2HPO_4$ (Panreac, a.r.), and $KH_2PO_4$ (Panreac, a.r.). NaOH (Merck, a.r). and HCl (Merck, a.r.) were also used to adjust the pH values in some cases. The total ionic strength was set to 150 mM.

## 5.2. Procedure

Three types of experiments were performed: acid-base titrations, melting experiments and mole-ratio studies. All of them were performed for oligonucleotide samples, ACTD samples and for the mixture of both. The experimental on-line set-up for spectroscopically monitored acid-base titrations has been described elsewhere [18]. The experimental conditions were 25 ºC and 150 mM ionic strength (147 mM $Na^{+}$, as NaCl, and 1 mM $Mg^{2+}$, as $MgCl_2 \bullet 6H_2O$). Titrations were carried out by adding increasing volumes of HCl stock solution to a slightly basic solution of oligonucleotide,

ACTD, or mixtures of oligonucleotide and ACTD at different ratios. CD and molecular absorption spectra were recorded at several pH values along the titration. Melting experiments were carried out from 16 to 84 ºC with a lineal temperature ramp of 0.8 ºC/min. CD and molecular absorption spectra were recorded every 3 $^{o}$C. The experimental conditions were 111 mM $Na^{+}$, 1 mM $Mg^{2+}$, 6 mM $KH_2PO_4$, 10 mM $Na_2HPO_4$, pH 6.9 (150 mM ionic strength). Each sample was allowed to equilibrate at the initial temperature for 30 min before the melting experiment was started. Mole-ratio experiments were carried out by progressive additions of small volumes of ACTD stock solution to a 1 ml oligonucleotide solution. The additions were done at time intervals of 15 minutes. The experimental conditions were the same as those described for melting experiments.

### 5.3. Instrumental

CD spectra between 220 and 600 nm were recorded on a Jasco J-810 spectropolarimeter equipped with Julabo F-25/HD temperature control unit. The software also allowed the recovery of molecular absorption spectra by means of a mathematical treatment of the output signal from the photomultiplier, allowing the simultaneous recording of molecular absorption and CD spectra. Hellma quartz cylindrical cuvettes (path length of 1.0 cm, 650 μl volume) were used. Alternatively, molecular absorption spectra were also recorded on a Perkin-Elmer lambda-19 spectrophotometer between 220 and 600 nm. In this case, temperature was controlled by a Perkin-Elmer peltier device. Hellma quartz cuvette (path length of 1.0 cm, 2000 μl volume) was used. pH measurements were performed with a CyberScan 2500 pH meter and a combined Hamilton pH electrode (Liq-Glass).

### 5.4. Data treatment

Thermodynamic information about each DNA sequence, ACTD, and their interaction was obtained from analysis of multivariate spectroscopic data. The information recovered from data recorded along an acid-base titration consisted on the number of acid-base species present in the studied pH range, their concentration profiles (defined by their corresponding $K_a$ values) and the pure spectra

for each one of those acid-base species. Similarly, the information recovered from data recorded along a melting experiment consisted on the number of conformations present in the studied temperature range, their concentration profiles (characterized by their melting temperatures, $T_m$) and the pure spectra for each one of them. Finally, for mole-ratio experiments, the information consisted on the number of species, including possible interaction complexes, their concentration profiles (defined by their formation constants, β) and their pure spectra.

CD spectra recorded along an experiment were arranged in a table or data matrix $\mathbf{D_{CD}}$. The dimensions of this matrix were *Nr* rows x $\lambda m$ columns, where *Nr* were the spectra recorded at successive pH, temperature or ACTD:oligonucleotide ratio values (depending on whether data from a titration, a melting or a mole ratio experiment was being analyzed), and $\lambda m$ was the number of wavelengths measured. Similarly, molecular absorption spectra were arranged in a data matrix $\mathbf{D_{abs}}$. When CD and molecular absorption spectra were simultaneously recorded, these were arranged in an augmented data matrix $[\mathbf{D_{CD}},\mathbf{D_{abs}}]$, the dimensions of which were *Nr* rows x $2*\lambda m$ columns.

Two different multivariate data analysis methods were applied to analyze the above described data matrices. Spectroscopy data recorded along acid-base titrations or mole-ratio experiments were analyzed by means of the hard-modeling method EQUISPEC [19]. Spectroscopic data recorded along melting experiments were analyzed with the soft-modeling MCR-ALS method [20, 21]. Both methods calculate the concentration profiles and the pure spectra for all spectroscopically active species present in the system from the decomposition of the experimental data matrix **D** according to equation:

$$\mathbf{D} = \mathbf{C}\,\mathbf{S}^{\mathbf{T}} + \mathbf{E} \qquad \text{(equation 1)}$$

where **C** and $\mathbf{S}^{\mathbf{T}}$ are respectively, the data matrices containing the concentration profiles and the pure spectra for each one of the species or conformations present in the experiment. **E** contains the residual noise not explained by the proposed species or conformations in **C** and $\mathbf{S}^{\mathbf{T}}$. Matrix **D** can be $\mathbf{D_{CD}}$, $\mathbf{D_{abs}}$ as well as the augmented data matrix $[\mathbf{D_{CD}},\mathbf{D_{abs}}]$.

EQUISPEC is a widely applied method for the determination of equilibrium constants, like $K_a$ (or $pK_a$) and β (or log β [19]). It is based on the postulation of an initial chemical model defined by: (1) the stoichiometries of the proposed species, (2) by approximate values of their equilibrium constants and (3) on the compliance of the mass-action law. In this work, the calculated log β values correspond to the equilibrium:

duplex + ACTD ↔ duplex:ACTD

where the DNA concentration is expressed as single strand. Logarithms of equilibrium constants are given as their weighted means with twice their standard errors (units of the least significant digit).

Hard-modeling methods, like EQUISPEC, are especially adequate for the study of chemical equilibria of monomers or oligomers, i.e., when mass-action law is fulfilled and no secondary effects related to large polymeric structures, like polyelectrolytic effects, polyfunctional effects or conformational changes are present [8]. Therefore, for large polymeric systems or when analyzing data from melting experiments, application of hard-modeling methods is rather difficult or even impossible since it is difficult to postulate a physical or chemical model describing the observed data variance. In these cases, however, data analysis is still possible by applying soft-modeling methods because they do not require the postulation or fulfillment of a particular physical or chemical model. Multivariate Curve Resolution using Alternating Least Squares (MCR-ALS) is a soft modeling method that has been widely applied for the study of acid-base and conformational transitions of polynucleotides [8, 18, 21, 22]. MCR-ALS consisted of the following steps:

1. Determination of the number of acid-base species or conformations, *N*: The number of spectroscopically active chemical species or conformations *N* was estimated by applying several methods like Evolving Factor Analysis (EFA) [23] or SIMPLISMA [24].

2. ALS optimization: The ALS optimization procedure is an iterative method used to solve the equation 1 for the proposed number of species or conformations *N*. This iterative process is started either with an initial estimate of the concentration profiles **C** or of the pure spectra $\mathbf{S^T}$, for each one of the *N* species or conformations proposed. The initial estimates of **C** or $\mathbf{S^T}$ were obtained from

EFA or SIMPLISMA, respectively. Along the ALS optimization, several constraints were applied to give chemical meaning to the mathematical solutions. Thus, molecular absorption data were constrained to be positive or zero. A similar constraint was applied to the calculated concentration profiles. For a more detailed explanation, the reader is refereed to previous works where the algorithm has been extensively explained [20]. Also, for examples of spectroscopic data analysis from complex systems, such as melting of cyclic oligonucleotides showing dumbbell-dimeric-random coil equilibria [21], or acid-base equilibria of Triplex Forming Oligonucleotides (TFO) [18].

All EQUISPEC and MCR-ALS calculations were performed using MATLAB routines (version 6, The Mathworks Inc, Natick, MA). MCR-ALS codes and tutorials are freely available at the URL: www.ub.es/gesq/mcr/mcr.htm.

## 6. Acknowledgments

We acknowledge the work performed by Alexandre Lekernec (*Institut Universitaire Technologique,* Lannion, France), Katarzyna Wrona (*Jagiellonian Univercity*, Cracow, Poland) and Roger Ramos (University of Barcelona). Prof. F.-M. Chen is specially thanked for his helpful comments. We acknowledge two grants from the Spanish *Ministerio de Educación y Ciencia* (projects BFU2004-02048/BMC and BQU2003-0191).

## 7. Table and figure captions

**Table 1**. Comparison of melting temperatures and formation constants for the sequences studied in this work and for their mixtures with ACTD.

**Scheme 1**. Chemical structure of actinomycin D.

**Figure 1.** Acid-base study of the oligonucleotide AGCT. (a) Experimental CD and molecular absorption data (matrix [$\mathbf{D_{CD}}$,$\mathbf{D_{abs}}$]). (b) Resolved concentration profile (matrix **C**) by application of EQUISPEC. (c) Resolved pure CD spectra for each one of the acid-base species present in the titration (matrix $\mathbf{S_{CD}}^{\mathbf{T}}$). (d) The corresponding resolved pure molecular absorption spectra (matrix $\mathbf{S_{abs}}^{\mathbf{T}}$). Oligonucleotide concentration: 5 μM. Other experimental conditions as detailed in the text. Red: acidic species. Green: neutral species. Blue: Basic species.

**Figure 2**. Melting study of the oligonucleotide GGCC_G. (a) Experimental CD and molecular absorption data (matrix [$\mathbf{D_{CD}}$,$\mathbf{D_{abs}}$]). (b) Resolved concentration profile (matrix **C**) by application of MCR-ALS. (c) Resolved pure CD spectra (matrix $\mathbf{S_{CD}}^{\mathbf{T}}$). (d) Resolved pure molecular absorption spectra (matrix $\mathbf{S_{abs}}^{\mathbf{T}}$). Oligonucleotide concentration: 15 μM. Other experimental conditions as detailed in the text. Green: native conformation. Blue: random coil conformation.

**Figure 3.** Melting study of ACTD. (a) Experimental CD and molecular absorption data (matrix $\mathbf{D_{CD}}$,$\mathbf{D_{abs}}$]). (b) Resolved concentration profile (matrix **C**) by application of MCR-ALS. (c) Resolved pure CD spectra (matrix $\mathbf{S_{CD}}^{\mathbf{T}}$). (d) Resolved pure molecular absorption spectra (matrix $\mathbf{S_{abs}}^{\mathbf{T}}$). ACTD concentration: 23 μM. Other experimental conditions as detailed in the text. Green: initial conformation. Blue: random coil conformation.

**Figure 4.** Mole-ratio study of the mixture GGCC_G:ACTD. (a) Experimental CD and molecular absorption data, matrix [$\mathbf{D_{CD}}$,$\mathbf{D_{abs}}$]. (b) Resolved concentration profiles, matrix **C**. (c) Resolved pure CD spectra, matrix $\mathbf{S_{CD}}^{\mathbf{T}}$. (d) The corresponding resolved pure molecular absorption spectra, matrix $\mathbf{S_{abs}}^{\mathbf{T}}$. Initial oligonucleotide concentration: 18 μM. ACTD concentration ranged from 0 to 33 μM. Other experimental conditions as detailed in the text. Red: interaction species. Blue: Neutral oligonucleotide. Green: ACTD.

**Figure 5.** Melting study of a mixture GGCC_G:ACTD. (a) Resolved concentration profile (matrix **C**) by application of MCR-ALS. (b) Resolved pure CD spectra (matrix $\mathbf{S_{CD}}^{\mathbf{T}}$). (c) Resolved pure molecular absorption spectra (matrix $\mathbf{S_{abs}}^{\mathbf{T}}$). (d) Residuals showing a random behavior (matrix **E**). Blue: mixture of the duplex:ACTD complex, free duplex and free ACTD. Cyan: mixture of the duplex:ACTD complex, free ACTD and unfolded free GGCC_G. Red: mixture of the hairpin:ACTD complex, free ACTD and unfolded free GGCC_G. Green: mixture of unfolded free GGCC_G and free ACTD.

**Table 1**. Comparison of melting temperatures and formation constants for the sequences studied in this work and for their mixtures with ACTD.

| Sequence | $T_m$ oligonucleotide [b] | log formation constant at 25 $^oC$, pH 6.9 | $T_m$ mixture |
|---|---|---|---|
| 5'-CAAAGCTTTG-3' | 36 | 5.1 ± 0.3 | 38, 55, 75 [c] |
| 5'-CATGGCCATG-3 | 49 | 6.4 ± 0.2 | 41, 53, 69 [d] |
| 5'-TATGGCCATA-3' | 43 | 5.6 ± 0.2 | 40, 46, 73 [e] |

[a] Values determined from multivariate analysis. The uncertainty associated to $T_m$ value was estimated ± 1 $^oC$.

[b] For a 5 microM oligonucleotide solution.

[c] For a 15 microM solution of oligonucleotide and 5 microM of ACTD.

[d] For a 12 microM solution of oligonucleotide and 9 microM of ACTD.

[3] For a 9 microM solution of oligonucleotide and 5 microM of ACTD.

**Scheme 1**. Chemical structure of actinomycin D.

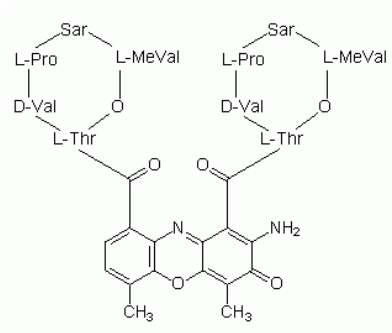


**Figure 1.** Acid-base study of the oligonucleotide AGCT. (a) Experimental CD and molecular absorption data (matrix [$\mathbf{D_{CD}}$,$\mathbf{D_{abs}}$]). (b) Resolved concentration profile (matrix **C**) by application of EQUISPEC. (c) Resolved pure CD spectra for each one of the acid-base species present in the titration (matrix $\mathbf{S_{CD}}^{\mathbf{T}}$). (d) The corresponding resolved pure molecular absorption spectra (matrix $\mathbf{S_{abs}}^{\mathbf{T}}$). Oligonucleotide concentration: 5 μM. Other experimental conditions as detailed in the text. Red: acidic species. Green: neutral species. Blue: Basic species.

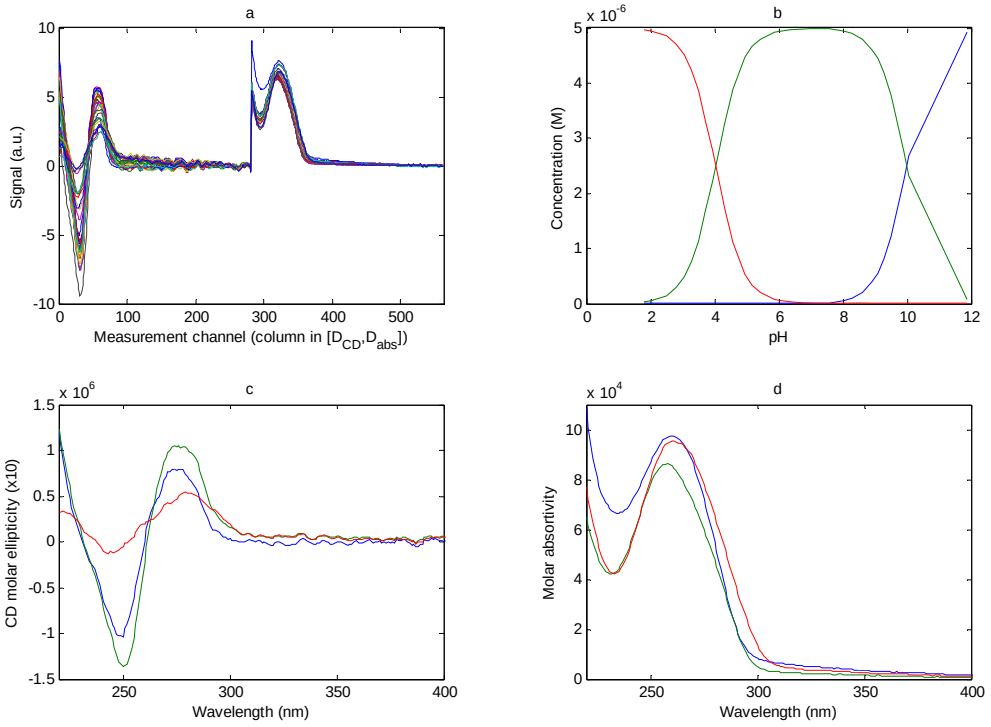


**Figure 2**. Melting study of the oligonucleotide GGCC_G. (a) Experimental CD and molecular absorption data (matrix [$\mathbf{D_{CD}}$,$\mathbf{D_{abs}}$]). (b) Resolved concentration profile (matrix **C**) by application of MCR-ALS. (c) Resolved pure CD spectra (matrix $\mathbf{S_{CD}}^{T}$). (d) Resolved pure molecular absorption spectra (matrix $\mathbf{S_{abs}}^{T}$). Oligonucleotide concentration: 15 μM. Other experimental conditions as detailed in the text. Green: native conformation. Blue: random coil conformation.

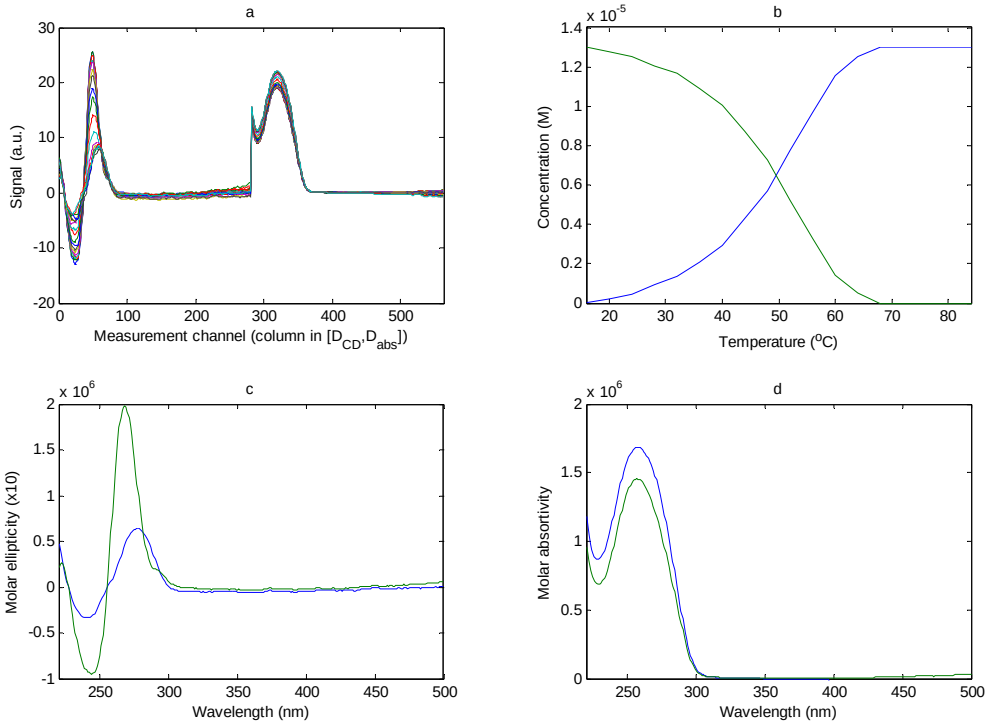


**Figure 3.** Melting study of ACTD. (a) Experimental CD and molecular absorption data (matrix $\mathbf{D_{CD},D_{abs}}$]). (b) Resolved concentration profile (matrix **C**) by application of MCR-ALS. (c) Resolved pure CD spectra (matrix $\mathbf{S_{CD}}^{\mathbf{T}}$). (d) Resolved pure molecular absorption spectra (matrix $\mathbf{S_{abs}}^{\mathbf{T}}$). ACTD concentration: 23 μM. Other experimental conditions as detailed in the text. Green: initial conformation. Blue: random coil conformation.

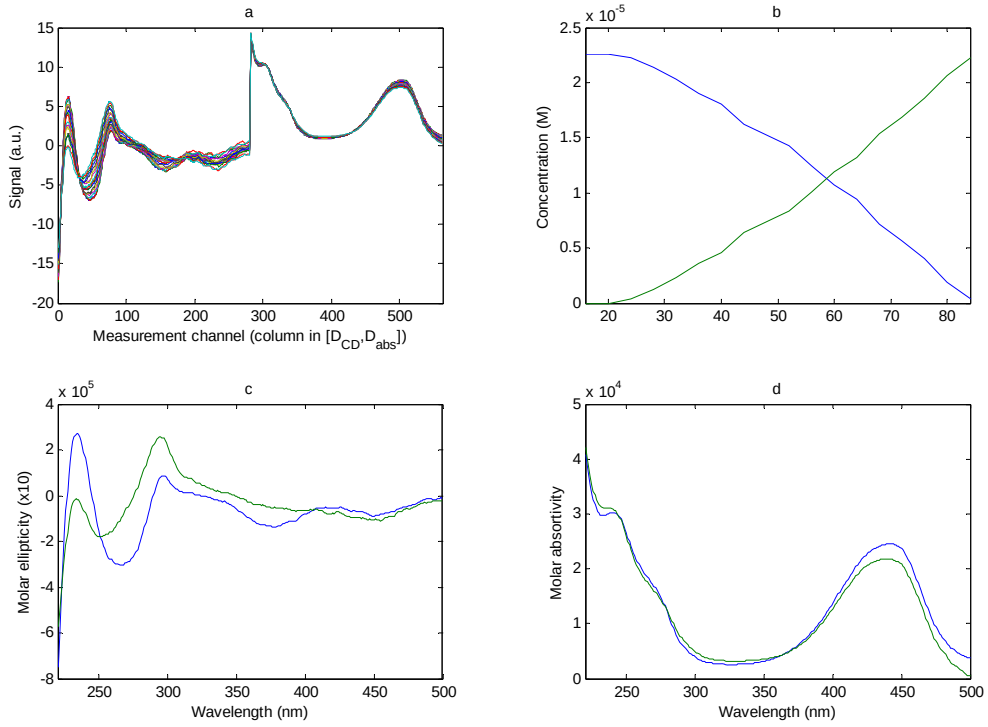


**Figure 4.** Mole-ratio study of the mixture GGCC_G:ACTD. (a) Experimental CD and molecular absorption data, matrix [$\mathbf{D_{CD}}$,$\mathbf{D_{abs}}$]. (b) Resolved concentration profiles, matrix **C**. (c) Resolved pure CD spectra, matrix $\mathbf{S_{CD}}^{\mathbf{T}}$. (d) The corresponding resolved pure molecular absorption spectra, matrix $\mathbf{S_{abs}}^{\mathbf{T}}$. Initial oligonucleotide concentration: 18 μM. ACTD concentration ranged from 0 to 33 μM. Other experimental conditions as detailed in the text. Red: interaction species. Blue: Neutral oligonucleotide. Green: ACTD.

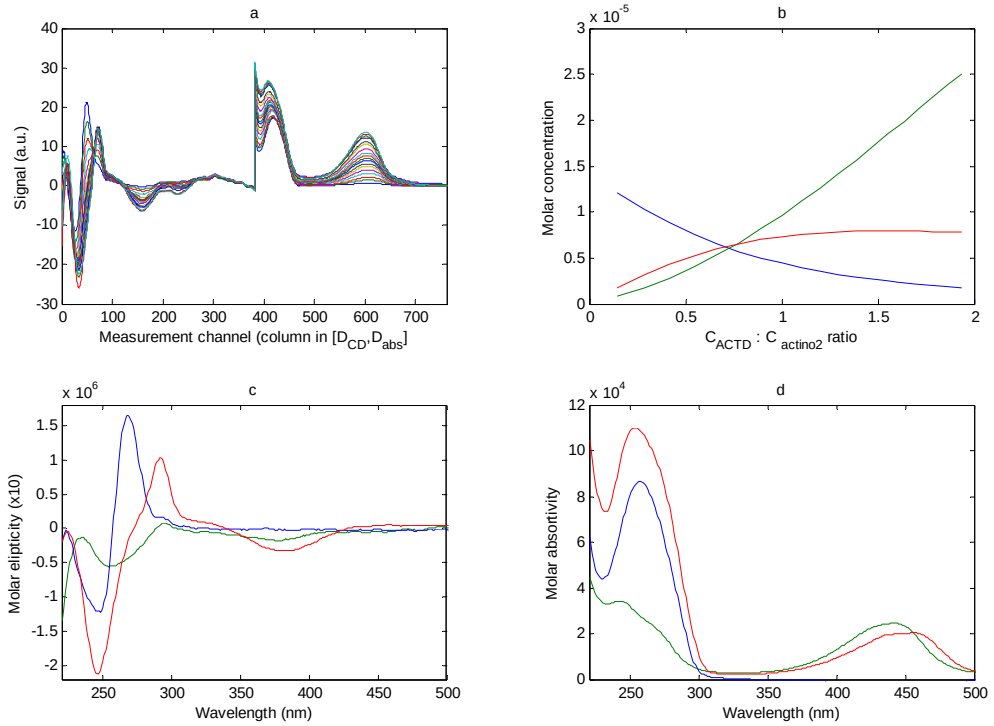
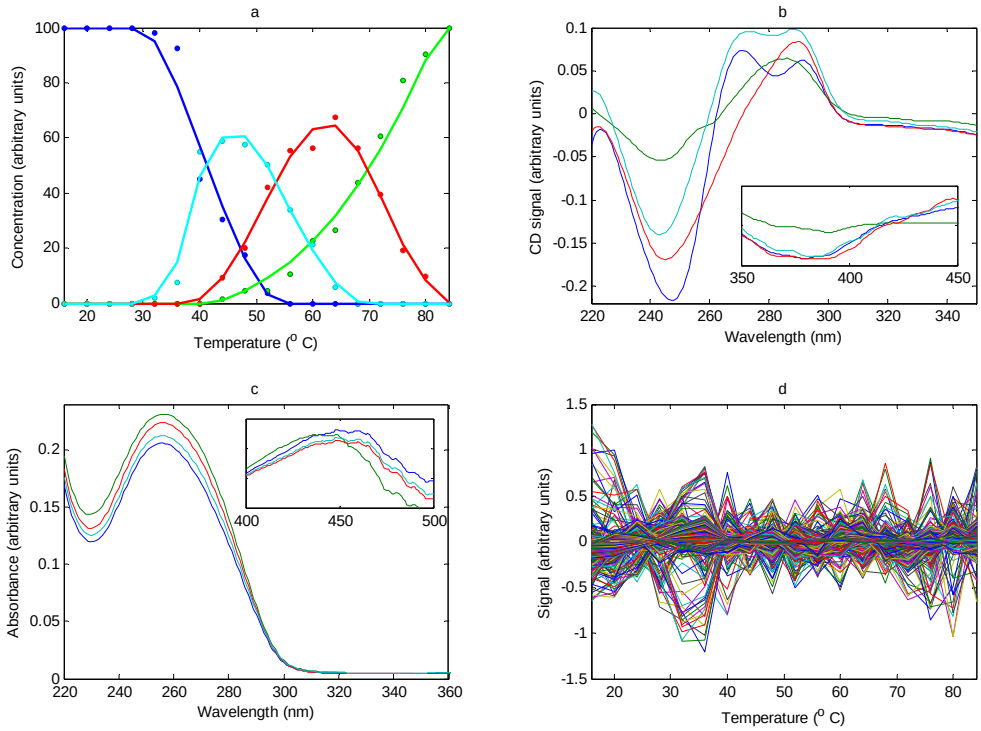


**Figure 5.** Melting study of a mixture GGCC_G:ACTD. (a) Resolved concentration profile (matrix **C**) by application of MCR-ALS. (b) Resolved pure CD spectra (matrix $\mathbf{S_{CD}}^{\mathbf{T}}$). (c) Resolved pure molecular absorption spectra (matrix $\mathbf{S_{abs}}^{\mathbf{T}}$). (d) Residuals showing a random behavior (matrix **E**). Blue: mixture of the duplex:ACTD complex, free duplex and free ACTD. Cyan: mixture of the duplex:ACTD complex, free ACTD and unfolded free GGCC_G. Red: mixture of the hairpin:ACTD complex, free ACTD and unfolded free GGCC_G. Green: mixture of unfolded free GGCC_G and free ACTD.



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